High-density lipoprotein (HDL) possesses key anti-atherogenic properties (3, 17, 24). In addition to reverse cholesterol transport and antioxidative activities, HDL exerts several beneficial effects on the vasculature including preventing and reversing endothelial dysfunction (10, 36, 49). These effects involve regulation of vascular tone and thrombogenicity by induction of cyclooxygenase-2 (COX-2) expression and prostacyclin I-2 (PGI-2) production in endothelial cells (28, 33).

Cyclooxygenase (COX) is a rate-limiting enzyme required for the conversion of arachidonic acid (AA) to eicosanoids (48). Of the two known COX isoforms, COX-1 is constitutively expressed under basal conditions in most tissues, whereas COX-2 is highly inducible and more often associated with inflammatory processes (48). Studies have shown that HDL could induce the expression of COX-2 in endothelial cells in a dose-dependent manner, which consequently facilitates the release of PGI-2 (16, 28, 33), a strong vasodilator that acts upon vascular smooth muscle cells and a potent inhibitor of platelet adhesion (26).

HDL is a complex of heterogeneous particle composed of various apolipoproteins, enzymes, and lipids. Those different components may exert diverse biological functions through different molecular mechanisms (13). Several groups have reported that HDL is a carrier of bioactive lysosphingolipids such as sphingosine-1-phosphate (SIP), sphingosylphosphorylcholine (SPC), and lysosulfatide (LSF) (32). In particular, SIP, which is predominantly integrated with HDL in plasma (~60% content), could play multiple roles in endothelial cells (2, 20, 22, 37), including the upregulation of COX-2 expression and the induction of PGI-2 release through SIP receptors (12, 21).

Apolipoprotein A-I (apoA-I), the major apolipoprotein of HDL, acts as an ATP binding-cassette transporter A1 (ABCA1)-dependent acceptor for cellular phospholipids (PL) and cholesterol during the biogenesis of HDL (45). In addition, apoA-I plays multiple roles in endothelial protection, such as inhibiting the expression of endothelial adhesion molecules and preventing apoptosis of endothelial cells (39, 42). Nevertheless, whether apoA-I is able to protect endothelial cells by activation of COX-2 expression and PGI-2 release still remains unknown. In the present study, we demonstrate that apoA-I, as another bioactive substance of HDL, could not only induce COX-2 expression and PGI-2 release but also enhance these effects with SIP in endothelial cells.
MATERIALS AND METHODS

Chemicals and reagents. The antibody to COX-2 (no. 160107), the antibody to COX-1 (no. 160110), SIP (no. 62570), and competitive enzyme immunoassay kit for 6-keto PGF\(_{1\alpha}\) (no. 515211) were purchased from Cayman Chemical (Michigan, IL). Pertussis toxin (PTX, Bordetella pertussis, BML-G100-0050) was purchased from Enzo Life Sciences International (Munich, Germany). The antibodies to phospho-extracellular regulated kinase (ERK)1/2 (T202/Y204, no. 4377), phospho-Janus kinase 2 (JAK2, Y1007/1008, no. 3776), ERK1/2 (no. 9102) and JAK2 (no. 3230) were purchased from Cell Signaling Technology (Danvers, MA). The antibodies to phospho-p38 mitogen-activated protein kinase (MAPK, Tyr 182, no. sc-7975-R) and to p38 MAPK (no. sc-7149) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal ABCA1 antibody (no. ab 18180) was purchased from Abcam (Cambridge, MA). Horseradish peroxidase (HRP)-goat-anti-rabbit IgG (no. MBL 458) and HRP-goat-anti-mouse IgG (no. MBL 350) were purchased from MBL (Nagoya, Japan). Human recombinant BSA (no. BSA 85) was p38 MAPK inhibitor SB203580 (no. S307), the ERK1/2 inhibitor PD98059 (no. P215), the JAK2 inhibitor AG490 (no. T3434), AA (no. A3555), and trypsin (proteomics grade, no. T6567) were purchased from Sigma-Aldrich (St. Louis, MO). Total cholesterol assay kit from Biosisino Bio-Technology (no. 00006090, Beijing, China), fatty acid assay kit from Beijing Sinopcr (no. SE1000, Beijing, China), and phospholipids assay kit from Kinghaw Pharmaceutical (no. PL7220, Beijing, China) were used to determine the components of HDL. Endothelial cell medium (ECM, no. 1001) was purchased from SclentCell Research Laboratories (Carlsbad, CA). Small interfering RNA (siRNA) specific for ABCA1 and scrambled control siRNA were synthesized by Shanghai GenePharm (Shanghai, China). Lipid-lipid interaction kit from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal ABCA1 antibody (no. ab18180) was purchased from Abcam (Cambridge, MA). Horseradish peroxidase (HRP)-goat-anti-rabbit IgG (no. MBL 458) and HRP-goat-anti-mouse IgG (no. MBL 350) were purchased from MBL (Nagoya, Japan). Human recombinant BSA (no. BSA 85) was purchased from ABCA1 (no. ab 18180) antibody (DiaSorin, Stillwater, OK) and quantified through the measurement of apoA-I content by nephelometry (Dimension XPand, Dade Behring, Germany). HDL was dialyzed with PBS, sterilized through a 0.22-μm filter, and stored in sealed tubes at 4°C in the dark for use within 1 mo.

The study protocol was approved by an Institutional Review Board of Peking University First Hospital (Beijing). Healthy volunteers were recruited and informed of the study, and written informed consent was obtained from all participants. Healthy volunteers must be healthy based on family history, laboratory, and physical exam.

Selective delipidation of HDL. HDL was selectively delipidated as described previously (8). Briefly HDL was agitated with diisopropyl ether in a ratio of 1:2 (vol/vol) for 24 h at 4°C. After extraction, the mixture was centrifuged at 2,000 rpm for 5 min to separate the aqueous and organic phases allowing for collection of apoHDL\&PL (HDL containing the protein components and the phospholipids) in the aqueous phase and PL-depleted HDL-lipids (the lipid components of HDL except for the phospholipids) in the organic phase. Alternatively, HDL was agitated with a mixture of butanol and diisopropyl ether (vol/vol) for 30 min at room temperature. After centrifugation we collected apoHDL (HDL only containing the protein components) in the aqueous phase and HDL-lipids (all HDL-lipid components) in the organic phase. ApoHDL\&PL was treated with the mixture of butanol and diisopropyl ether to purify HDL-PL (the phospholipids of HDL). ApoHDL\&PL and apoHDL were filtered before cell treatment. PL-depleted HDL-lipids, HDL-PL, and HDL-lipids were dried under nitrogen on ice and processed as described previously (7), and 0.5% fatty acid-free BSA was used as a carrier to substitute for apoA-I.

Agarose gel electrophoresis. Agarose gel electrophoresis of HDL was carried out as described previously (31). Briefly, 200 μl of HDL (1 mg/ml), apoHDL\&PL, and apoHDL were incubated with 20 μl of Sudan black B staining solution at 37°C for 30 min and then centrifuged at 2,000 rpm for 5 min. Agarose gels at 0.6% (wt/vol) in 50 mM barbital buffer (pH 8.6) were cast in a Bio-Rad Mini Sub gel electrophoresis system. A sample (50 μl) was loaded in each lane and was subjected to electrophoresis at room temperature for 1 h at 150 V in barbital buffer.

Isolation of apoA-I from HDL. Delipidated HDL as described above was subjected to 12% SDS-PAGE under nonreducing conditions. After staining was completed, the gel slices containing apoA-I were cut, electroeluted, dialyzed, and lyophilized as described previously (19). ApoA-I was analyzed by 12% SDS-PAGE and Western blot using goat anti-apoA-I polyclonal antibody.

Digestion of apoA-I by trypsin. The trypsin digestion procedure was carried out according to the manufacturer’s protocol. The lyophilized trypsin (proteomics grade) was reconstituted in 1 mM HCl at a concentration of 1 mg/ml (20 μl of 1 mM HCl for each vial with 20 μg trypsin). ApoA-I was dissolved in 100 mM ammonium bicarbonate (pH 8.5). Trypsin at a ratio of 1:50 (wt/wt) was added to the apoA-I solution. The samples were then incubated for 18 h at 37°C, dialyzed with phosphate-buffered saline (PBS) for 48 h, and sterilized again for further use.

Western blot analysis. HUVECs were washed twice with ice-cold PBS and then lysed with RIPA buffer. Equal amounts of total protein were loaded onto SDS-PAGE gels and blotted onto the nitrocellulose membrane. Subsequently, blots were blocked with 5% nonfat milk in TBS with 0.1% Tween-20 for 1 h and incubated with respective primary antibodies. The blots were probed with either HRP-goat-anti-rabbit IgG or HRP-goat-anti-mouse IgG secondary antibodies. The specific immunoreactive protein bands were detected by ECL and visualized using Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA).

Quantitation of 6-keto PGF\(_{1\alpha}\) by competitive ELISA. To measure 6-keto-PGF\(_{1\alpha}\) production, HUVECs were exposed to different kinds of HDL (30 μg/ml) or apoA-I (10 μg/ml) for 6 h, washed twice with PBS, and then incubated with 0.75% fatty acid-free BSA/PBS buffer containing exogenous AA (10 μmol/l) for 30 min in an incubator with 5% CO\(_2\) at 37°C (33, 41). Each sample of 50 μl of was processed for PGII-2 release assay according to the manufacturer’s instructions.
within 12 h. Cells were lysed with RIPA buffer and the protein concentration was assayed by a bicinchoninic acid (BCA kit, Pierce, Rockford, IL) method for correcting the ELISA data (pg of 6-keto PGF$_1\alpha$/mg of cellular protein). Data are means ± SE of 3 separate experiments.

siRNA transfection. For transfection with siRNA, HUVECs were plated in six-well plates ($1 \times 10^6$ cells/well). At 30–50% confluence, HUVECs were transfected with 100 nM of ABCA1 siRNA or scrambled control siRNA (Opti-MEM) and Lipofectamine RNAiMAX according to the manufacturer’s protocol. After transfection for 6 h, the medium was replaced by ECM with ECGS and 5% FBS. The target sequence for human ABCA1 siRNA was sense 5’-GAUUCACCCACCAACGAGAATT-3’ and anti-sense 5’-UGGGACCCGAUUAGAAGCTT-3’. And the sequence for scrambled control siRNA was sense 5’-UUCUCCGAACGUGACACGU-3’ and anti-sense 5’-ACGUAGACGUUCCAGGAATT-3’. After 48 h incubation and 6 h of serum deprivation, HUVECs were induced with 10 μg/ml of apoA-I for 6 h to assay COX-2 expression and PGI-2 release.

Statistical analysis. All experiments were repeated at least three to four times. Data are presented as means ± SE. Differences were compared with two-tailed Student’s t-test or one-way ANOVA using GraphPad Prism (5.0) software. P < 0.05 was considered statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001).

RESULTS

ApoA-I, the major protein component of HDL, induces COX-2 expression and PGI-2 release in HUVECs. To investigate whether apoA-I could induce COX-2 expression and PGI-2 release, we disassembled HDL particle through different delipidation processes as described in MATERIALS AND METHODS. ApoA-I was also isolated from HDL as described in MATERIALS AND METHODS. As anticipated, HDL upregulated COX-2 expression in HUVECs treated for 6 h (Fig. 1, A and B) and induced PGI-2 release (Fig. 1C). The concentration of HDL was 517.2 ± 45.15 pg/ml (P < 0.01). After delipidation, apoHDL&PL and apoHDL still induced COX-2 expression (Fig. 1, A and B) and PGI-2 production (Fig. 1C, apoHDL&PL vs. PBS: 517.2 ± 45.15 vs. 291.5 ± 18.31 pg/ml, P < 0.01; apoHDL vs. PBS: 390.3 ± 27.63 vs. 291.5 ± 18.31 pg/ml, P < 0.05). As the main apolipoprotein of HDL, apoA-I can induce COX-2 expression (Fig. 1, A and B) and facilitate PGI-2 release alone (Fig. 1C, apoA-I vs. PBS: 394.8 ± 5.815 vs. 291.5 ± 18.31 pg/ml, P < 0.01). In striking contrast, apoA-II, the second most abundant HDL protein did not have these capabilities (Fig. 1, A–C). Moreover, similar to HDL (Fig. 1D), apoA-I induced COX-2 expression in time- (0.5–8 h) and dose-dependent (1–60 μg/ml) manners in HUVECs (Fig. 1, E and F). Accordingly, apoA-I bound to HUVECs in a time- and dose-dependent manner (see online Supplement Fig. 1, A and B, at the AJP-Cell Physiol website). In addition, the purity of isolated apoA-I was noted to be excellent (see online Supplement Fig. 2). Also, by treating phorbol 12-myristate 13-acetate (PMA)-activated THP-1 cells with 10 μg/ml of apoA-I for 4 h and 1 μg/ml of lipopolysaccharide (LPS) as a positive control to measure the release of TNF-α and IL-1β, we excluded the possibility that apoA-I was contaminated by LPS (Supplement Fig. 3, A and B).

Trypsinized apoA-I loses its ability to induce COX-2 expression and PGI-2 release. To further confirm that the effects of COX-2 expression and PGI-2 release were triggered by apoA-I, we digested apoA-I with proteomics-grade trypsin, which can also digest the enzyme itself, and we treated HUVECs with the trypsinized apoA-I to measure COX-2 expression and PGI-2 release. ApoA-I and the trypsinized apoA-I were assayed by 12% SDS-PAGE and stained with Coomassie brilliant blue R-250. ApoA-I was completely degraded by trypsin (Fig. 2A). Importantly, we found that trypsinized apoA-I cannot upregulate COX-2 expression (Fig. 2, B and C) nor facilitate PGI-2 release (Fig. 2D) in HUVECs, confirming that intact apoA-I is required for the upregulation of COX-2 expression and PGI-2 release.

Phospholipid components of HDL upregulate COX-2 expression in HUVECs. To detect whether HDL lipids can independently induce COX-2 expression, we collected different types of lipids from HDL. HUVECs were treated for 6 h with HDL-PL, HDL-lipids (including HDL-PL), and PL-depleted HDL-lipids (HDL-lipids without phospholipids), the desired components of which were equal to that of 30 μg/ml of HDL. Exogenous S1P (2 μM) was used as a positive control in the same experiment. HDL-PL, HDL-lipids, and S1P significantly upregulated COX-2 expression in HUVECs (Fig. 3). However, 0.5% BSA, cholesterol (20 μg/ml), and PL-depleted HDL-lipids had little effect on the induction of COX-2 expression (Fig. 3). Therefore, the bioactive phospholipids of HDL, including S1P, play a key role in the induction of COX-2 in HUVECs.

ApoA-I enhances COX-2 expression and PGI-2 release with S1P in HUVECs. To investigate whether apoA-I and S1P would exert additive effects on the induction of COX-2 expression and PGI-2 release, we treated HUVECs for 6 h with 0.5% BSA, S1P (2 μM of S1P carried by 0.5% BSA), apoA-I (10 μg/ml), S1P-apoA-I (2 μM of S1P carried by 10 μg/ml of apoA-I), and HDL (30 μg/ml) to measure COX-2 expression and PGI-2 release. S1P and apoA-I upregulated COX-2 expression (Fig. 4A and B) and facilitate PGI-2 release (Fig. 4C, S1P vs. BSA: 762.6 ± 44.67 vs. 345.9 ± 38.33 pg/ml, P < 0.001; S1PApoA-I vs. BSA: 688.7 ± 49.85 vs. 345.9 ± 38.33 pg/ml, P < 0.01). Interestingly, S1P-apoA-I treatment led to an even greater increase than incubation with S1P or apoA-I alone (Fig. 4A, S1P-apoA-I vs. S1P: 978.4 ± 11.22 vs. 762.6 ± 44.67 pg/ml, P < 0.01). S1PApoA-I vs. S1P: 978.4 ± 11.22 vs. 688.7 ± 49.85 pg/ml, P < 0.01). To determine whether PTX, an inhibitor of S1P receptors, could inhibit the ability of HDL, S1P, or apoA-I to induce COX-2 expression, we pretreated HUVECs with or without PTX (200 ng/ml) for 24 h and subsequently incubated HUVECs with 30 μg/ml of HDL, 2 μM of S1P (S1P carried by 0.5% BSA), or 10 μg/ml of apoA-I for 6 h. PTX significantly inhibited the expression of COX-2 induced by S1P and partially inhibited the effects of HDL. However, PTX did not effectively inhibit COX-2 expression induced by apoA-I (Fig. 4D and E). Collectively, these data strongly support that apoA-I can synergistically enhance the effects of S1P through distinct pathways.

COX-2 expression and PGI-2 release induced by HDL or apoA-I are inhibited significantly by different inhibitors in HUVECs. To investigate the intracellular molecular mechanisms involved in COX-2 expression and PGI-2 production induced by HDL or apoA-I, we pretreated HUVECs with the p38 MAPK inhibitor SB203580 (1 μM), the ERK1/2 inhibitor
PD98059 (10 μM), or the JAK2 inhibitor AG490 (20 μM) for 1 h, respectively, and then HUVECs were subsequently incubated with HDL (30 μg/ml) or apoA-I (10 μg/ml) for 6 h to assay COX-2 expression and PGI-2 production. SB203580, and AG490 significantly inhibited COX-2 expression (Fig. 5, A and B) and PGI-2 release induced by HDL (Fig. 5C, HDL vs. HDL + SB203580: 1,168 ± 41.27 vs. 145.4 ± 12.94 pg·ml⁻¹·mg cellular protein⁻¹, P < 0.001; HDL vs. HDL + PD98059: 1168 ± 41.27 vs. 238.6 ± 17.26 pg·ml⁻¹·mg cellular protein⁻¹, P < 0.001; HDL vs. HDL + AG490: 1168 ± 41.27 vs. 555.5 ± 51.44 pg·ml⁻¹·mg cellular protein⁻¹, P < 0.001). Moreover, SB203580, PD98059, and AG490 also inhibited COX-2 expression (Fig. 5, D and E) and PGI-2 production induced by apoA-I (Fig. 5F, apoA-I vs. apoA-I + SB203580: 837.5 ± 74.34 vs. 435 ± 52.3 pg·ml⁻¹·mg cellular protein⁻¹, P < 0.05; apoA-I vs. apoA-I + PD98059: 837.5 ± 74.34 vs. 140.9 ± 6.944 pg·ml⁻¹·mg cellular protein⁻¹, P < 0.001; apoA-I vs. apoA-I + AG490: 837.5 ± 74.34 vs. 214.3 ± 28.9 pg·ml⁻¹·mg cellular protein⁻¹, P < 0.01). These data support the hypothesis that p38 MAPK, ERK1/2, and JAK2 pathways mediate the effects of HDL and apoA-I.

**Activation of signaling pathways involved in COX-2 expression and PGI-2 release induced by HDL or apoA-I.** HUVECs were stimulated with 30 μg/ml of HDL (Fig. 6A) or 10 μg/ml of apoA-I (Fig. 6B) at different time points, and the activation of p38 MAPK, ERK1/2, and JAK2 was detected by Western blot analysis. Protein samples were immunoblotted with anti-phospho-p38 MAPK, anti-total-p38 MAPK, anti-phospho-ERK1/2, anti-total-ERK1/2, anti-phospho-JAK2, anti-total-JAK2, and anti-β-actin antibodies. Time-course experiments showed that p38 MAPK, ERK1/2, and JAK2 were activated with maximal phosphorylation occurring between 5 and 60 min. Representative bands from three independent experiments were shown and the quantitation was summarized in online Supplement Fig. 4. These data suggest the activation of key components of p38 MAPK, ERK1/2, and JAK2 pathways in response to HDL and apoA-I treatment in endothelial cells.

**ABCA1 knockdown inhibits COX-2 upregulation and PGI-2 production induced by apoA-I.** To investigate whether ABCA1 was involved in the induction of COX-2 and PGI-2 by apoA-I, we silenced the expression of ABCA1 by siRNA. The amount of 200 μg of total protein was loaded onto a 6% SDS-PAGE gel, and the downregulation of ABCA1 protein was detected using Western blot analysis. Our data showed that the siRNA sequence effectively downregulated the expression of ABCA1 in HUVECs (Fig. 7A). ApoA-I could not upregulate the expression of COX-2 (Fig. 7, B and C) and the release of PGI-2

![Fig. 1. Apolipoprotein A-I (ApoA-I) induced cyclooxygenase-2 (COX-2) expression and prostacyclin-2 (PGI-2) release in human umbilical vein endothelial cells (HUVECs). A–C] HUVECs were incubated with phosphate-buffered saline (PBS), high-density lipoprotein (HDL) (30 μg/ml), HDL containing the protein components and the phospholipids (PL) (apoHDL&PL) (30 μg/ml), HDL only containing the protein components (apoHDL) (30 μg/ml), apoA-I (10 μg/ml), and apoA-II (10 μg/ml) for 6 h, and the expression of COX-2 and COX-1 was assayed by Western blot analysis (A) and the production of PGI-2 was determined by competitive ELISA (C). The relative protein expression was normalized to β-actin (B). D: HUVECs were induced by different doses of HDL for 6 h, and the expression of COX-2 and COX-1 was assayed by Western blot analysis. E: HUVECs were incubated with different doses of apoA-I for 6 h, and the expression of COX-2 and COX-1 was assayed by Western blot analysis. F: HUVECs were incubated with apoA-I (10 μg/ml) at different time points, and the expression of COX-2 and COX-1 was assayed by Western blot analysis. Data were means ± SE from three separate experiments. *P < 0.05 vs. PBS group, **P < 0.01 vs. PBS group, ***P < 0.001 vs. PBS group. ns, not significant.
Selective delipidation of HDL preserves the desired components of HDL efficaciously. To identify whether the selective delipidation of HDL effectively removed the target components of HDL but preserved the desired effects of HDL, we assayed the content of total cholesterol, free fatty acid, phospholipids, total protein, and apoA-I in HDL, apoHDL&PL, and apoHDL. The electrophoretic mobility of HDL, apoHDL&PL, and apoHDL was detected by agarose gel electrophoresis. Proteins of HDL, apoHDL&PL, and apoHDL were separated by 12% SDS-PAGE and stained with Coomassie brilliant blue R-250. When compared with HDL, total cholesterol and free fatty acid were substantially depleted in apoHDL&PL (77.8% removal for total cholesterol and 81.9% removal for free fatty acid) and the content of phospholipids was decreased by 20.1%.

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fatty acid), but the phospholipid and total protein content remained similar in apoHDL&PL and HDL. Similarly, for apoHDL, 90.37% of total cholesterol, 82.28% of free fatty acid, and 64.7% of phospholipids were removed from HDL, but the total protein components were constant in apoHDL compared with HDL (Fig. 8, A–D). The electrophoretic mobility of apoHDL&PL was similar with that of native HDL, whereas the electrophoretic mobility of apoHDL was a little faster than that of native HDL (Fig. 8E). Noticeably, both the total protein and apoA-I content were effectively preserved in apoHDL&PL and apoHDL (Fig. 8F).

DISCUSSION

In the present study, we demonstrated for the first time that apoA-I, one of the bioactive components of HDL, induced the expression of COX-2 and the production of PGI-2 through the p38 MAPK, ERK1/2, and JAK2 pathways via ABCA1 in endothelial cells, and apoA-I also strengthened these effects with S1P.

Numerous studies have reported that PGI-2 produced from endothelial cells has beneficial effects against atherogenesis including promotion of vasorelaxation, inhibition of leukocyte-endothelial cell adhesion, and platelet aggregation (26, 30). Importantly, selective COX-2 inhibitors increased the risk of cardiovascular events, which might be caused by the inhibition of PGI-2 production in endothelial cells (18, 35). Previous studies have shown that HDL exerts protective effects on endothelial cells both in vitro and in vivo, including the induction of PGI-2 production (5, 29, 36). In the early studies, the salutary effects of HDL were attributed to provision of AA, the precursor for PGI-2 (14, 38). Moreover, HDL could also upregulate COX-2 expression and promote PGI-2 release through alternate intracellular pathways in endothelial cells (28, 33).

It is important to note that HDL is a complex composed of apolipoproteins, enzymes, and lipids, and each component could have distinct effects on endothelial cell. The complexity of the signaling pathways modulated by HDL also reflects the possibility of differential effects of the individual components of HDL on the gene expression of endothelial cells and the subsequent manipulation of endothelial function (13, 34, 46). Several studies indicated that S1P, a key bioactive lysosphingolipid mainly integrated in HDL, could induce COX-2 expression and PGI-2 release in endothelial cells (12, 20, 21, 37). Our results also showed that HDL-PL and HDL-lipids, which include S1P and other phospholipids of HDL, could significantly upregulate COX-2 expression in endothelial cells, but PL-depleted HDL-lipids have little effects (Fig. 3). Besides S1P, SPC and LSF are also present in HDL-PL and could exert...
multiple biofunctions through S1P receptors, such as activating endothelial nitric oxide (NO) synthase and facilitating NO production in endothelial cells (32). Therefore, we speculate that SPC and LSF could also induce COX-2 expression and PGI-2 release through S1P receptors. Indeed, Pomerantz et al. (38) indicated that delipidated HDL could also enhance PGI-2 production but to a lower extent than native HDL, which suggested that apolipoproteins of HDL could account for the remaining effect. Additional studies also support this view that the protein component of HDL can have effect on PGI-2 production (5, 29). However, the exact protein component of HDL that is responsible for this effect was unclear. Therefore, we tested whether apoA-I, the major apolipoprotein of HDL, may be the bioactive component of HDL to induce the expression of COX-2 and the release of PGI-2 in endothelial cells.

Fig. 5. COX-2 expression and PGI-2 release induced by HDL or apoA-I were inhibited by different inhibitors in HUVECs. A–F: HUVECs were pretreated with 30 μg/ml of HDL or 10 μg/ml of apoA-I at different time points, and the activation of p38 MAPK, ERK1/2, and JAK2 was analyzed by Western blot analysis. Protein samples were immunoblotted with anti-phospho-p38 MAPK, anti-total-p38 MAPK, anti-phospho-ERK1/2, anti-total-ERK1/2, anti-phospho-JAK2, anti-total-JAK2, and anti-β-actin antibodies. Representative bands from three independent experiments were shown.

Fig. 6. Phosphorylation of signaling pathways involved in COX-2 expression and PGI-2 release induced by HDL or apoA-I in HUVECs. A and B: HUVECs were incubated with 30 μg/ml of HDL or 10 μg/ml of apoA-I at different time points, and the activation of p38 MAPK, ERK1/2, and JAK2 was analyzed by Western blot analysis. Protein samples were immunoblotted with anti-phospho-p38 MAPK, anti-total-p38 MAPK, anti-phospho-ERK1/2, anti-total-ERK1/2, anti-phospho-JAK2, anti-total-JAK2, and anti-β-actin antibodies.
Our results show that apoHDL&PL, apoHDL, and apoA-I (but not apoA-II) induce COX-2 expression and PGI-2 release in endothelial cells (Fig. 1). In addition to upregulating COX-2 expression, HDL could also induce the phosphorylation of caveolin-1, thus promoting PGI synthase shuttling from the membrane to the perinuclear area, which could result in the coupling of COX-2 with PGI-synthase and the more production of PGI-2 (33). However, whether apoA-I has this effect on caveolin-1 still needs further study. Moreover, apoA-I...
strengthened the effects with S1P to induce COX-2 expression and PGI-2 release (Fig. 4), which suggests that apoA-I exerts these effects through a complimentary mechanistic. Studies have shown that the upregulation of COX-2 and the production of PGI-2 induced by S1P could be abrogated by pertussis toxin (PTX), an irreversible inhibitor of Gαi/Gαo proteins (12, 16, 21, 28), which is consistent with our results. However, these effects induced by apoA-I were not inhibited by PTX, and those induced by HDL were only partially inhibited by PTX (Fig. 4, D and E), which suggests a different mechanism of apoA-I.

Several groups have demonstrated that the interaction of apoA-I with ABCA1 exerts crucial anti-inflammatory effects via activation of JAK2/STAT3 pathway (44). The activation of the JAK2/STAT3 pathway induced by ischemia preconditioning upregulates COX-2 expression and protects cardiomyocytes against myocardial ischemia-reperfusion injury (4). Therefore, we speculated that apoA-I could upregulate COX-2 expression through the JAK2/STAT3 pathway in endothelial cells. Here, activation of the JAK2/STAT3 pathway was shown using a JAK2 inhibitor (Fig. 5) and immunoblotting analysis (Fig. 6B). In addition, apoA-I could promote lipid removal and decrease intracellular cholesterol content through the activation of JAK2 via ABCA1 (45). Cholesterol depletion of caveolae and low cell cholesterol levels could both activate p38 MAPK and ERK1/2 pathways (6, 15). Smith et al. (41) also demonstrated that increasing the concentration of LDL or free cholesterol decreased COX-2 expression and PGI-2 synthesis, suggesting that cellular cholesterol balance plays an important role in determining COX-2 levels. Thus we hypothesize that the interaction of apoA-I with ABCA1 may result in the activation of p38 MAPK and ERK1/2 pathways by decreasing the intracellular cholesterol concentration, thereby upregulating COX-2 expression in endothelial cells. Accordingly, the results of our experiments using inhibitors of p38 MAPK and ERK1/2 (Fig. 5) showed that apoA-I also induced COX-2 expression and PGI-2 release through p38 MAPK and ERK1/2 pathways, in agreement with observed changes in phosphorylation of p38 MAPK and ERK1/2 (Fig. 6B). Aiello RJ et al. (1) and Tang et al. (44) showed that either downregulation or mutation of ABCA1 impaired the effects of lipid removal and anti-inflammation effects of apoA-I. We demonstrated that siRNA knockdown of ABCA1 can inhibit the expression of COX-2 and the release of PGI-2 (Fig. 7) and the phosphorylation of p38 MAPK, ERK1/2, and JAK2 (Supplement Fig. 5) induced by apoA-I, which confirmed the central role of ABCA1 in mediating the effects of apoA-I.

Studies have indicated that HDL induces COX-2 expression by activating the p38 MAPK and ERK1/2 pathways (25, 33), yet whether the JAK2 pathway was involved in these effects remains unclear. Several studies have shown that HDL exerts many biological effects through S1P receptors and that S1P could activate the JAK2 pathway through S1P receptors (23, 32, 47). Moreover, HDL inhibits the activation of nuclear factor-κB induced by tumor necrosis factor-α in endothelial cells, but HDL synergizes with tumor necrosis factor-α to enhance the expression of COX-2 and the synthesis of PGI-2, implying that the JAK2/STAT3 pathway is possibly involved in the expression of COX-2 in endothelial cells (4, 11). Therefore, we investigated whether HDL may activate JAK2 pathway through S1P receptors. Importantly, our results show that the JAK2-specific inhibitor AG490 could inhibit the effects of HDL on COX-2 expression and PGI-2 release (Fig. 5), and that JAK2 could be phosphorylated by HDL confirming that activation of this pathway may mediate the effects of HDL.

In summary, the major finding of this study is that the interaction of apoA-I with ABCA1 functions to protect endothelial cells through increased COX-2 expression and PGI-2 release by the activation of the p38 MAPK, ERK1/2, and JAK2 pathways. These results not only further reinforce the protective mechanism of apoA-I in endothelial cells but also suggest that lipid-free apoA-I, as well as HDL, could partially decrease the cardiovascular risk caused by COX-2 inhibitors through the upregulation of COX-2 expression and PGI-2 release in endothelial cells. Although epidemiological studies have suggested that HDL-C levels correlate with the plasma concentration of 6-keto PGF1α, the stable PGI-2 metabolite (40, 43), further studies are required to confirm a causal role.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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